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Oocyte Maturation in the Brachiopod *Terebratalia transversa:* Role of Follicle Cell-Oocyte Attachments During Ovulation and Germinal Vesicle Breakdown

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Abstract. In the brachiopod *Terebratalia transversa,* each ovarian follicle consists of (i) a prophase-arrested oocyte with an enlarged nucleus (= the germinal vesicle, or GV), and (ii) a surrounding sheath of follicle cells that attach to the oocyte by means of junctional complexes. Within about 80 min after removing a follicle from the ovary, the follicular sheath undergoes a microfilament-mediated retraction, and the ovulated oocyte that emerges from the retracted sheath subsequently completes germinal vesicle breakdown (GVBD). Based on the experimental manipulations reported here, it appears that the follicle must be detached from the ovary for such ovulation and GVBD to occur. Moreover, GVBD can be prevented if the oocytes are mechanically stripped of their follicle cells up to 30 min after being isolated from the ovary. GVBD proceeds normally, however, if follicle cells are removed more than 40 min after the follicle is obtained from the ovary. The percentage of oocytes that undergo GVBD is also diminished following treatment with drugs that uncouple gap junctions. Collectively, these data suggest that removing a follicle from the ovary stimulates follicle cells to produce a maturation-inducing factor that uses the follicle cell–oocyte junctional complexes to reach the oocyte within about 30–40 min after follicular removal. The significance of these findings is discussed relative to previous reports on oocyte maturation in brachiopods and other animals.

Introduction

In many groups of animals, oocytes develop in close association with a surrounding sheath of follicle cells as part of an integrated unit called a follicle (Raven, 1961). During differentiation of a typical follicle, the oocyte nucleus (= the germinal vesicle, or GV) becomes greatly enlarged while the oocyte remains arrested in prophase I of meiosis. In addition, projections of the follicle cells also can attach to the oolemma of the oocyte by means of gap junctions that allow small molecules to pass between the follicle cells and the prophase-arrested oocyte (Anderson and Albertini, 1976; Patino and Purkiss, 1993; York *et al.,* 1993). When triggered by the proper stimulus, the oocyte resumes meiotic maturation by initiating germinal vesicle breakdown (GVBD) and ultimately emerges from its follicular sheath during a process referred to as ovulation.

As noted in various investigations (Eppig, 1985; Kwon *et al.,* 1990; Schultz, 1991; Downs, 1997), maturation depends on a dynamic interplay between oocytes and their surrounding follicle cells. For example, follicle cells can secrete maturation-inducing substances that presumably use the junctions between follicle cells and oocytes to promote GVBD and ovulation (Schroeder, 1981; Beers and Olsiewski, 1985; Sato and Koide, 1987; Cerada *et al.,* 1993). On the other hand, mammalian follicle cells may initially produce substances that inhibit maturation; this conclusion is based on the observation that competent oocytes undergo spontaneous maturation if removed from their follicular sheaths (Pincus and Enzmann, 1935) and on subsequent confirmations of follicle-cell-derived inhibition in experimental analyses (Cho *et al.,* 1974; Racowsky, 1985). More recent investigations, however, suggest that mammalian follicle cells also supply a maturation-inducing substance that overcomes the effects of the previous inhibition of maturation (Downs *et al.,* 1988; Downs, 1995).

Thus, follicle cells can stimulate or inhibit maturation, and such regulatory signals may be transported through the junctions at the follicle cell–oocyte interface. To supplement our current understanding of the roles of follicle

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cells during oocyte maturation, additional studies of a broader range of animals are needed.

Brachiopods constitute a comparatively small phylum of sessile or sedentary marine invertebrates that possess a calcified shell and a ciliated lophophore used in filter feeding (Hyman, 1959; Williams and Rowell, 1965; Rudwick, 1970). The roughly 120 genera of living brachiopods are traditionally grouped into two classes—the Inarticulata and Articulata—which are distinguished on the basis of differences in shell structure, anatomy, and reproductive biology (James *et al.,* 1992).

Recent reviews of brachiopod reproduction and embryology provide relatively little information about oocyte maturation (Chuang, 1983; Reed, 1987; Long and Stricker, 1991). Since those reviews were published, thorough investigations have been conducted on the process of oogenesis in the articulate brachiopod *Terebratulina retusa* (James *et al.,* 1991a, b; 1992) and on the endocrine pathway leading to oocyte maturation in the inarticulate species *Glottidia pyramidata* (Freeman, 1994). However, neither the precise relationship between ovulation and GVBD nor the possible roles played by follicle cell–oocyte attachments during maturation have yet been defined for brachiopods.

In the investigation reported here, time-lapse video microscopy, electron microscopy, and experimental manipulations were used to analyze *in vitro* oocyte maturation in the articulate brachiopod *Terebratalia transversa.* The results suggest that (i) follicles need to be detached from the ovaries for ovulation and GVBD to occur and (ii) within 30–40 min after follicular detachment from the ovaries, oocyte maturation is triggered by a maturation-inducing substance that is transferred from the follicle cells to the oocyte via junctional complexes connecting follicle cells to the oolemma. The results of this investigation are compared to previously published data on brachiopods, and the events observed during *in vitro* maturation are discussed with respect to how oocyte maturation may be accomplished during normal development in the field.

Materials and Methods

Collection of animals and handling of gametes

Adult specimens of *Terebratalia transversa* (Sowerby, 1846) measuring 20–40 mm in shell width were collected by dredging or scuba diving in the vicinity of San Juan Island, Washington. Most females obtained during the summer had incompletely developed oocytes that did not successfully undergo maturation or fertilization, whereas fully gravid specimens occurred more commonly during the peak spawning season in the winter. Thus, to enhance the numbers of ripe specimens for studies of oocyte maturation, intact females were stored for several months in laboratory aquaria with running seawater so that, as previously noted by Reed (1987), developmentally competent oocytes could accumulate in the ovaries.

Observations and experiments were conducted during May to August, 1995 and 1996, or from December 1996 to February 1997. Specimens used in this study had moderately to fully ripe ovaries, and at least 50% levels of ovulation and GVBD were attained after oocyte maturation was triggered *in vitro.* In cases where ovaries contained mixed stages of oogenesis, relatively small oocytes that were incapable of undergoing maturation were ignored; the dataset thus included only well-developed specimens that measured >100 μm in diameter and occurred within a prominent follicular sheath situated at least 5 μm from the surface of the oocyte; *i.e.,* those essentially corresponding to "stage IV oocytes" in the four-stage classification scheme of Long (1964).

For analyses of oocyte maturation, the bivalved shell of each adult female was pried apart with a scalpel, and the inner surfaces of the shell valves were scraped with a glass pipette to dislodge follicles from the ovarian tissue that lay near the shell. Follicles that were isolated from ripe ovaries by such maceration techniques were rapidly washed once with filtered seawater (SW) and subsequently kept at 12°–16°C.

Microscopy

For light microscopic observations, isolated follicles that had been obtained from macerated ovaries were placed in specimen dishes maintained at 12°–16°C by a thermoelectric cooling stage (Stricker, 1994). Oocyte maturation was then monitored by video microscopy, using an inverted microscope, CCD (charge-coupled device) video camera, and a time-lapse video recorder set at 70–120× time-compression (Stricker *et al.,* 1994a). Alternatively, maturation was also examined by means of either a Nikon Optiphot photomicroscope equipped with 35-mm camera or a Bio-Rad MRC-600 confocal microscope employing nonconfocal, transmitted-light optics (Stricker *et al.,* 1994b).

To verify the patterns observed in living specimens, cultures of isolated follicles were incubated at 12°–16°C and serially fixed in formalin/glutaraldehyde (Stricker, 1995). Some of the fixed specimens were subsequently treated with a Triton- and glycerol-containing solution to render the oocytes more translucent (Stricker and Schatten, 1989), whereas nuclear architectural changes were monitored in follicles that had been labeled with the DNA-binding dye Hoechst 33342 (Sigma) prior to fixation (Stricker, 1995).

For transmission electron microscopy (TEM), pieces of ovaries or isolated oocytes were doubly fixed in glutaraldehyde and osmium tetroxide, dehydrated in ethanol, and embedded in "LX-112" Epon equivalent (Ladd Research Co.) (Stricker *et al.,* 1992a). After 1-μm sections

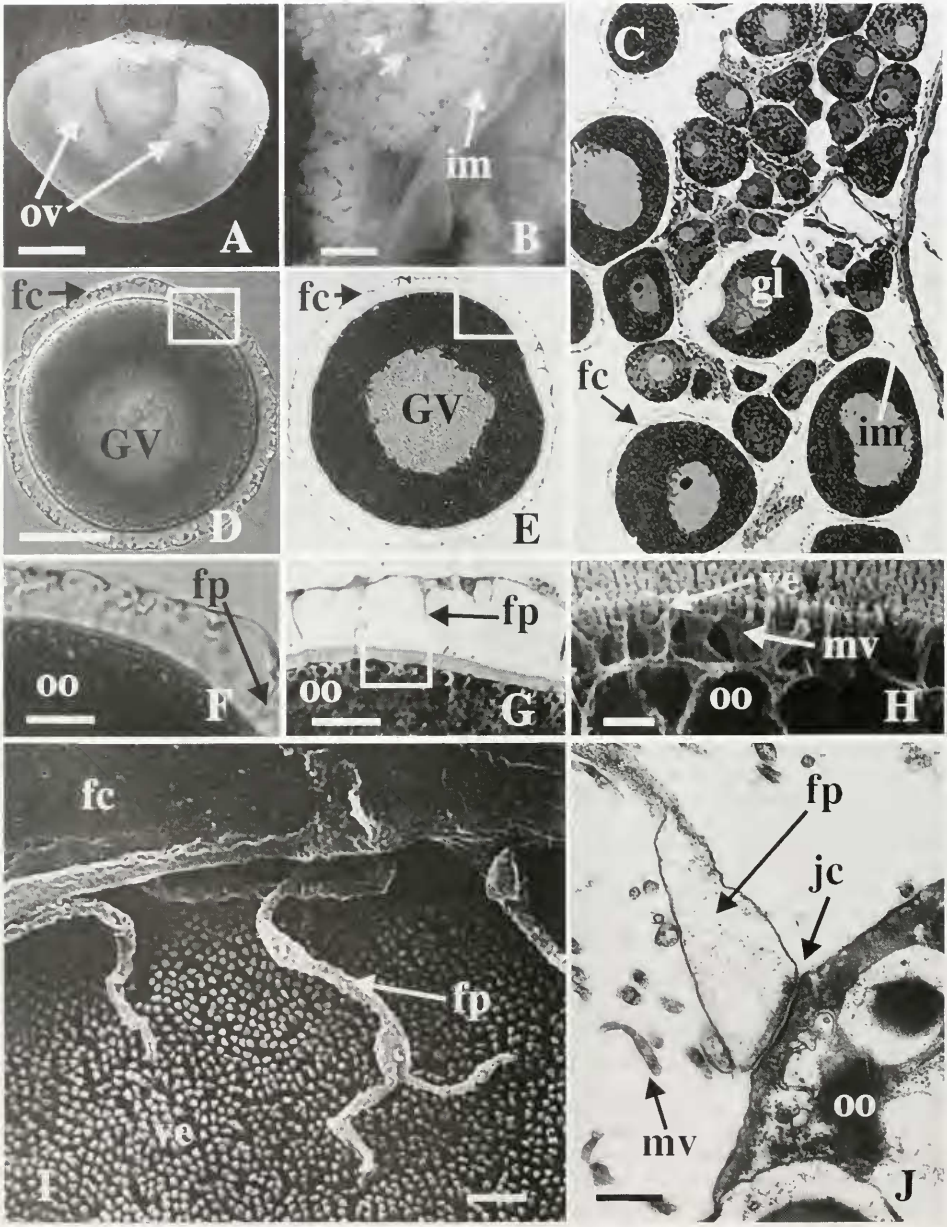


Figure 1. (A) Video macrograph of the dorsal valve of a ripe *Terebratalia transversa* female with the lophophore and viscera removed to show the U-shaped ovarian lobes (ov) that lie between the inner and outer mantle membranes underlying the shell; scale bar = 5 mm. (B) Video macrograph, showing that after

were obtained for orientation, the embedded specimens were sectioned at 70 nm and stained with uranyl acetate followed by lead citrate (Stricker *et al.,* 1992a). For scanning electron microscopy (SEM), isolated follicles or follicle-cell-free oocytes were fixed in a bicarbonate-buffered solution of osmium tetroxide, dehydrated in ethanol, dried by the critical-point method, and coated with gold-palladium (Stricker and Cloney, 1983).

Video images were digitized using an Image-1 image processing system (Universal Imaging Corp.), whereas EM or 35-mm negatives were printed, and the prints were subsequently digitized at 500 dpi using a UMAX S8 flatbed scanner. Some of the digital files were subjected to an "unsharp mask" or a "sharpening" kernel to enhance details before being constructed into montages using the MetaMorph software of Universal Imaging Corp. The montages were then labeled with CorelDraw 7.0 and recorded on 4X5 T-Max 100 film using an Agfa QZR-C film recorder (Stricker *et al.,* 1994b).

Experimental manipulations

To test the effects of mechanically removing follicle cells, a piece of Nitex (85–95 μm mesh) was glued over the cut front end of a 5-ml syringe to construct a cylindrical filter. At various time-points after maceration of the ovaries, the isolated follicles were drawn through the Nitex-covered syringe 5–10 times so that nearly all of the oocytes were completely stripped of their follicle cells without significantly damaging the oocytes.

For analyses of microfilament-mediated processes, isolated follicles were incubated in a SW solution of the actin-depolymerizing agent cytochalasin D (CD) (Sigma) that was prepared from a 10 m*M* stock mixture in dimethylsulfoxide (DMSO) (Sigma). One drop (~50 μl) of follicles was added to 1.5 ml of 10 μ*M* CD, based on previous findings that 10 μ*M* solutions of cytochalasin inhibited ovulation and GVBD in starfish oocytes (Schroeder, 1971; Stricker and Schatten, 1991). Controls received 0.1% DMSO without CD.

To determine the effects of altering the adhesion of follicle cells to oocytes, isolated follicles were incubated in calcium-free seawater (CaFSW) (Schroeder and Stricker, 1983) or a 1% seawater solution of trypsin (Sigma). For the CaFSW treatments, follicles were gently washed with numerous changes of CaFSW to ensure that external calcium concentrations were negligible.

Alternatively, seawater solutions of drugs that uncouple the gap junctions formed by various cell types (Davidson *et al.,* 1986; Sandberg *et al.,* 1990; Downs, 1995; Germain and Anctil, 1996) were applied to isolated follicles to test whether intercellular communication via such junctions is required for normal maturation. The drugs were purchased from Sigma and consisted of 1 m*M* heptanol or 25 μ*M* 18-α-glycyrrhetinic acid (AGA), based on the effective levels used on other oocytes (Patino and Purkiss, 1993; Downs, 1995). The AGA solutions were prepared from a 5 m*M* stock made in DMSO, and 0.5% DMSO was added to the control treatments.

For experimental analyses, oocytes were collected from at least two females, and the experiments were typically repeated three or more times. To assess the percentages of maturation and the sizes of oocytes, 60–200 oocytes were examined per ovary or timepoint of data collection. Statistical analyses involved Student's *t* tests, or Mann-Whitney *U* tests for large and small datasets, respectively (Sokal and Rohlf, 1973). Sample sizes for the timing of maturation processes varied, since not all events could be clearly discerned in each specimen.

Results

Morphology of the ovaries and prophase-arrested oocytes

In gravid females, a bilobed, U-shaped ovary occurred between the inner and outer mantle membranes underly-

the inner mantle membrane (im) is reflected with fine forceps to expose a ripe ovary, the follicles (arrows) remain attached to the genital lamellae of the ovary; scale bar = 500 μm. (C) Epon section (1 μm) of an incompletely ripe ovary, showing oocytes at various stages of oogenesis attached to the genital lamellae (gl) that arise from the inner mantle (im); well-developed oocytes are surrounded by a sheath of follicle cells (fc); scale bar = 50 μm. (D) Photomicrograph of an isolated follicle obtained after macerating a ripe ovary; fc = follicle cells; GV = germinal vesicle; the rectangle outlines a region similar to that shown at higher magnification in 1F; scale bar = 50 μm. (E) Epon section (1 μm) of an isolated follicle, showing the surrounding follicle cells (fc) and the centrally located germinal vesicle (GV); the rectangle outlines a region similar to that shown at higher magnification in 1G; scale bar = 50 μm. (F) Transmitted-light image, obtained using a confocal microscope, of follicle-cell projections (fp) that attach to the oolemma of fully developed oocytes (oo); scale bar = 10 μm. (G) Epon section (1 μm) of the follicle-cell projections (fp) that attach to the surface of the oocyte (oo); the rectangle outlines a region similar to that shown at higher magnification in 1H; scale bar = 10 μm. (H) Scanning electron micrograph (SEM) of a fractured oocyte (oo) that is no longer surrounded by a follicular sheath. The vitelline envelope (ve) comprises an interconnected meshwork of filaments at the apices of the oocyte's microvilli (mv); scale bar = 1 μm. (I) SEM of the follicle cell projections (fp) that penetrate the vitelline envelope (ve) to attach to the oocyte via junctional complexes; fc = follicle cells; scale bar = 1 μm. (J) Transmission electron micrograph of a follicle cell projection (fp) and the junctional complex (jc) it makes with the oolemma of the oocyte (oo); mv = microvilli; scale bar = 0.5 μm.

ing the dorsal and ventral valve of each shell (Fig. 1A, B). Such ovarian lobes consisted of a series of folds, or "genital lamellae" (James *et al.*, 1991a), that were covered throughout by a germinal epithelium, to which clusters of developing oocytes and fully formed follicles were connected (Fig. 1C). After the inner mantle was gently reflected with fine forceps to uncover a ripe ovary, the exposed gonad routinely maintained an intact outline (Fig. 1B), suggesting that the follicles remained attached to the germinal epithelium and were not freely floating in the space between the inner and outer mantle.

Before the onset of oocyte maturation, each follicle comprised a prophase-arrested oocyte and its enveloping sheath of follicle cells. The diameter of fully grown oocytes averaged 131 ± 10 μm in 15 specimens examined, and the size of such oocytes did not differ markedly in ventral *versus* dorsal ovaries or in winter *versus* summer. Each well-differentiated oocyte contained a centrally located GV that had a single large nucleolus and involuted nuclear envelope (Fig. 1D, E). Many branched microvilli arose from the surface of the oocyte and were interconnected at their apices by a meshwork of crosslinked filaments that collectively constituted an extremely hardy vitelline envelope (Fig. 1F–H).

The follicular sheath surrounding each prophase-arrested oocyte consisted of roughly two dozen flattened, nonciliated cells with tightly joined boundaries. In fully differentiated follicles corresponding to stage IV of Long (1964), the follicle cells were situated about 10 μm from the surface of the oocyte and extended many slender projections toward the oolemma (Fig. 1D–G). These follicle-cell projections penetrated the vitelline envelope (Fig. 1I) and connected with the oolemma by means of junctional complexes (Fig. 1J) that resembled the junctions reported for other marine invertebrate follicles (Anderson, 1969; Schroeder *et al.*, 1979; Schroeder, 1981).

Triggers of in vitro *maturation*

As noted previously (Long, 1964; Reed, 1987; Freeman, 1993), *in vitro* maturation can be triggered simply by macerating ripe ovaries of *T. transversa*. Fully formed follicles that were isolated by this method completed ovulation and GVBD in tandem, with ovulated oocytes undergoing GVBD, and GVBD failing to occur in oocytes that did not ovulate. In five ovaries containing relatively small oocytes (avg. diameter = 114 ± 8 μm), ovulation and GVBD occurred in less than 5% of the isolated follicles examined. Such nonmaturing oocytes did not develop upon insemination, whereas relatively high percentages (>80%) of normal development were achieved if oocytes were fertilized following GVBD (pers. obs.).

To assess which parameters of the maceration procedure were necessary for triggering oocyte maturation, a few concentrated drops of isolated follicles were placed in 10 ml of filtered SW within 30 s of ovarian maceration, and the follicles were rapidly washed again with 10 ml of SW. In all nine trials, washed oocytes continued to mature at the same levels as observed in follicles maintained in the macerated ovarian fluids (pers. obs.). This result indicates that any soluble substances released by the maceration procedure were not continuously required for maturation or were not diluted to subthreshold levels by the double washes in seawater. Conversely, to determine maturation levels in nonmacerated ovaries exposed to seawater, isolated shell valves with intact ovaries were immersed in seawater, whereas the ovarian lobes from the other half of each animal were macerated to trigger oocyte maturation. After being incubated for 3 h, only 9% ± 8% of the oocytes aspirated with a pipette from intact ovaries (n = 7) had matured, but a significantly higher percentage (92% ± 10%; n = 7; $P < 0.05$) of the follicles isolated from macerated ovaries had matured. This result indicates that the simple exposure of follicles to seawater cannot fully account for the high levels of *in vitro* maturation that are achieved by disrupting ovarian tissues.

To test for possible maturation-inducing substances released by the ovaries during maceration, shell valves with intact ovaries were placed upright in bowls and treated with 1–2 ml of either filtered SW or an "ovarian supernatant" that was freshly obtained by macerating the ovary from the other half of the animal in 1–2 ml of seawater, and then centrifuging the macerated tissues and fluids at 14,000 × *g* for 1 min to produce a cell-free supernatant. These solutions were either injected beneath the inner mantle with a 25-gauge syringe, or the valves were cut in half with a scalpel so that the ovarian lobe on each half-valve could be immersed in the test solution. After 3 h of incubation, follicles were aspirated from the ovaries with a pipette, and while doing so, care was taken to avoid any oocytes that had become dislodged during handling of the ovaries. As is evident in Table I, treatments with either seawater or ovarian supernatant failed to trigger significant levels of maturation in intact ovaries by 3 h post-treatment. The slightly elevated maturation percentages compared to the 9% ± 8% average obtained in previous seawater treatments of intact ovaries could be attributed simply to the presence of more detached oocytes (dislodged by the injections or valve bisections) than in seawater incubations of intact valves.

In other experiments involving more prolonged incubations, the ovarian tissue on one shell valve was macerated to provide both an ovarian supernatant and a set of control follicles that were detached from the ovaries. The other shell valve was then bisected with a scalpel so that one ovarian lobe was immersed in the ovarian supernatant, while the other intact lobe was covered with seawater. After 20 h of incubation, the number of mature oocytes in the intact ovaries treated with the supernatant more

Table I

Percentages of mature oocytes in intact ovaries vs. detached follicles of Terebratalia transversa

Duration of treatment (h)	n[3]	Intact ovaries[1]		Detached follicles[2]
		Seawater	Ovarian supernatant	Controls
3	5	19 ± 29%	23 ± 35%	91 ± 7%*
20	3	17 ± 12%	59 ± 27%**	96 ± 6%**

[1] For experimental treatments, an intact ovarian lobe on a bisected shell valve was immersed in either seawater or a supernatant obtained from macerated ovarian tissues. Alternatively, a single whole shell valve with intact ovarian lobes was given a submantle injection of either seawater or the ovarian supernatant. After 3 or 20 h of treatment, oocytes were aspirated from the ovaries, and the percentage of maturation was assessed.

[2] Controls consisted of detached follicles that were obtained from the macerated ovarian tissues used for the production of the ovarian supernatant. Maturation rates were assessed in these isolated follicles 3 or 20 h after ovarian maceration.

[3] Number of specimens in each treatment or control.

* Significantly greater than the seawater or supernatant treatments ($P < 0.05$).

** Significantly greater than the seawater treatment ($P < 0.05$).

closely approached the values obtained in the macerated controls (Table I). Conversely, the percentage of maturation in intact ovaries treated with seawater was not as high as in the supernatant trials (Table I).

Timing and patterns of ovulation and germinal vesicle breakdown

After detachment from the ovary, the follicular sheath of more than 90% of the fully grown follicles examined underwent a dynamic retraction to form a cap of aggregated follicle cells at one pole of the oocyte (Fig. 2A–H). In individual specimens monitored by time-lapse video microscopy at 12°–16°C, cap formation occurred 81 ± 15 min (n = 71) after detachment of the follicles from the ovaries (Fig. 3). This timing was also corroborated by serial fixations of mass cultures that interpolated the time at which 50% of the culture had undergone follicle-cell capping (= "$T_{1/2}$ ovulation") as 77 ± 20 min (n = 7).

Follicle-cell projections started to withdraw from the oolemma 19 ± 9 min (n = 47) before the cap was formed, and follicle-cell capping was accomplished in 8 ± 3 min (n = 30) from the onset of a poleward movement to the formation of a distinct cap. After ovulation was completed, the exposed vitelline envelope contained numerous holes that presumably represented the former sites of follicle-cell penetrations through the envelope, and the caps eventually became dislodged from the oocytes (Fig. 2I). Whether a preferred site of cap formation existed relative to the animal-vegetal axis of the oocyte was not determined, because the GV tended to be centrally located in the oocyte and follicle-cell caps were not present at polar body formation to provide landmarks for orientation.

In six batches of oocytes fixed 3 h after detachment of the follicles from the ovaries, GVBD occurred in 92% ± 9% of the isolated follicles. Based on time-lapse video analyses of living specimens, GVBD began with the disassembly of the nuclear envelope 8 ± 3 min (n = 13) after the first noticeable withdrawal of follicle-cell projections from the oolemma. Accordingly, incompletely ovulated oocytes contained a GV with indistinct boundaries (Fig. 2J), and in three fixation series, the time at which 50% of the specimens had already begun GVBD ("$T_{1/2}$ GVBD onset") averaged 70 ± 12 min after removal of the follicles from the ovaries *versus* a $T_{1/2}$ ovulation time of 80 ± 9 min post-removal.

After nuclear envelope disassembly, the nucleoplasm mixed with the surrounding cytoplasm, and the nucleolus eventually disintegrated to mark the end of GVBD (Fig. 2K, L), which in turn occurred 24 ± 16 min after the completion of ovulation (n = 6). By 3 h after removal of the follicle from the ovary, oocytes that had originally contained a GV and distinct nucleolus (Fig. 4A) possessed a circular array of chromosomes at the metaphase plate (Fig. 4B). Following insemination, such post-GVBD oocytes routinely cleaved and developed into normal larvae (Reed, 1987; pers. obs.).

Experimental manipulations of ovulation

To determine the effects of inhibiting ovulation, washed follicles were immersed in SW solutions of cytochalasin D within 5 min after detachment of the follicles from the ovaries. Following 3 h of incubation, 10 μM CD blocked follicle-cell capping in more than 90% of the oocytes, whereas 1 μM CD inhibited ovulation in less than 5% of the follicles (pers. obs.; Fig. 4C–G). This inhibition was reversed by washing CD-treated follicles in SW (Figs. 4F, G; 5), and ovulation was verified in time-lapse video studies of the washed specimens (pers. obs.), suggesting that rather than killing follicle cells, the CD treatment temporarily inhibited follicle-cell capping by disrupting microfilament-mediated movements; such disruption has been noted for other marine invertebrates (Schroeder, 1971; Smiley and Cloney, 1985).

After about 1 h in the CD solution, the flattened follicle cells rounded up, became separated by large gaps in what was previously a contiguous follicular sheath, and acquired spindly projections with knoblike endings (Fig. 4C–E). However, GVBD still proceeded in these morphologically altered follicles, as was apparent from images of intact specimens and sectioned material (Fig. 4C, D). In comparison to the more than 90% ovulation and GVBD obtained for control specimens not receiving CD, an average of 79% ± 9% of the CD-treated specimens

had undergone GVBD by 3 h post-incubation, but only 8% ± 17% of these oocytes were devoid of follicle cells in 17 trials using 10 μM CD. Thus, the CD treatments significantly ($P < 0.05$) reduced the percentage of ovulation, but not that of GVBD.

In contrast, isolated follicles that were treated with CaFSW to accelerate, rather than to inhibit, ovulation routinely retained their full complement of follicle cells directly after washing, and they subsequently completed ovulation and GVBD (Figs. 4H). However, as seen in four sets of serial fixations, CaFSW significantly accelerated the rate of ovulation ($P < 0.05$) and slightly decreased the time before the onset of GVBD, compared to control cohorts that were incubated in SW (CaFSW: $T_{1/2}$ ovulation = 60 ± 19 min; $T_{1/2}$ GVBD onset = 65 ± 18 min *vs.* SW: $T_{1/2}$ ovulation = 79 ± 21 min; $T_{1/2}$ GVBD onset = 69 ± 17 min) (Fig. 6). Treatments with 1% seawater solutions of trypsin, which caused follicle cells to lie close to the oolemma (Fig. 4I), also altered maturation kinetics as observed following CaFSW treatments (Trypsin: $T_{1/2}$ ovulation = 61 ± 3 min; $T_{1/2}$ GVBD onset = 60 ± 11 min *versus* SW: $T_{1/2}$ ovulation = 80 ± 13 min; $T_{1/2}$ GVBD onset = 73 ± 11 min). After being washed in SW and subsequently inseminated, such CaFSW- or trypsin-treated specimens developed into larvae (Fig. 4J) resembling those that could be successfully triggered to metamorphose (Stricker and Reed, 1985), collectively indicating that ovulation could be accelerated without preventing normal development.

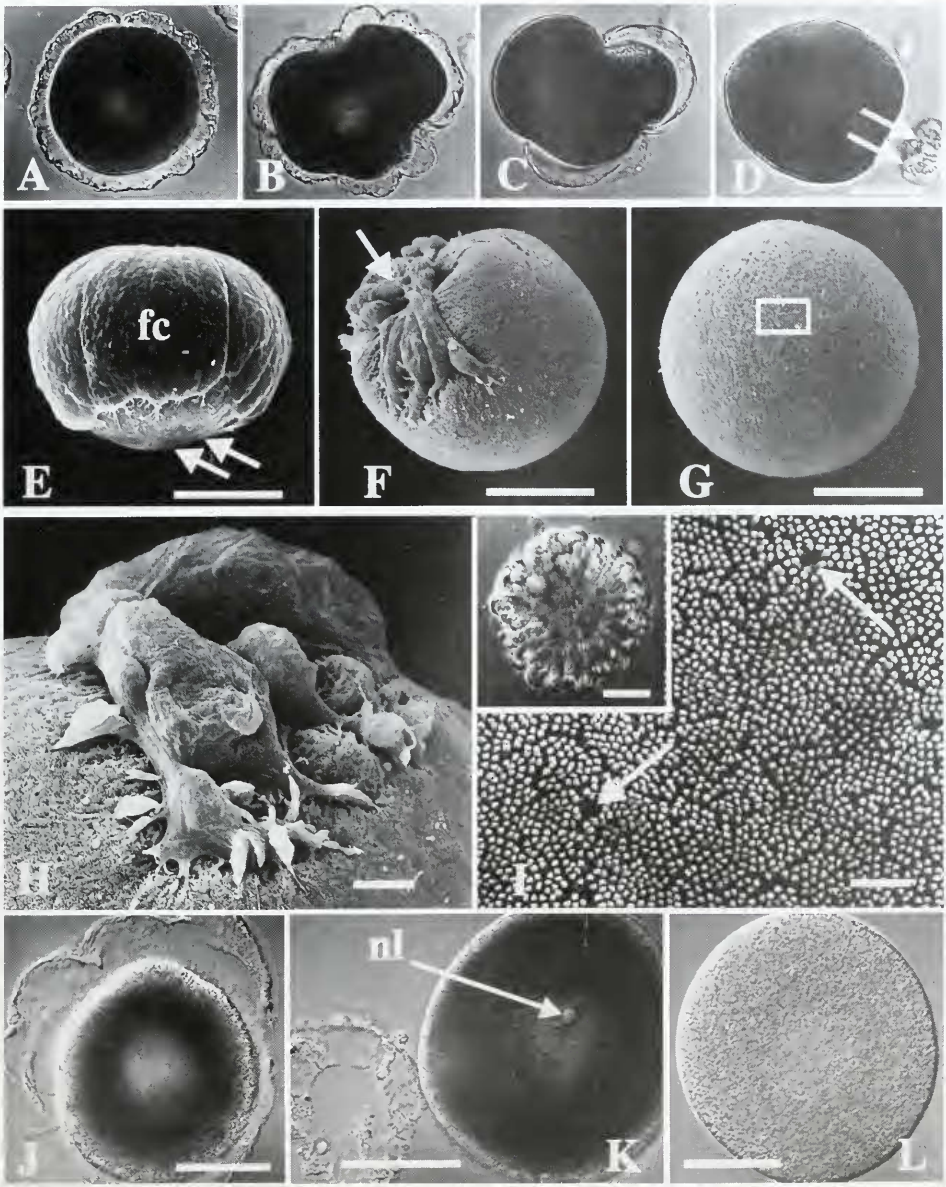
Similarly, for undetermined reasons, oocytes obtained from ventral ovaries completed ovulation more rapidly than did cohort oocytes collected from dorsal ovaries (ventral ovaries: $T_{1/2}$ ovulation = 73 ± 11 min; dorsal ovaries: 98 ± 19 min; n = 5; $P < 0.05$). Moreover, by macerating only one ovarian lobe on each half of the shell and then allowing the other lobe to be pre-incubated in seawater for at least 3 h before being macerated, the rates of ovulation were significantly faster in the pre-incubated specimens than in the non-incubated controls (pre-incubated ovaries: $T_{1/2}$ ovulation = 59 ± 14 min; non-incubated ovaries: 84 ± 12 min; n = 8; $P < 0.05$).

Effects of altering follicle cell–oocyte attachments during oocyte maturation

To determine whether follicle cells need to be attached to the oocyte for GVBD to occur, a Nitex filter was used to separate oocytes from their surrounding follicular sheaths at various timepoints following ovarian maceration (Fig. 7A). If follicle cells were stripped up to 30 min after detachment of the follicles from the ovaries, very few of the follicle-cell-free oocytes completed GVBD (Figs. 7B, 8), and the block was essentially permanent, because in all eight cultures that had been stripped of follicle cells at 5 min post-removal from the ovary, the levels of GVBD remained lower than 10% when examined the next morning. Such precocious removals of follicle cells inhibited GVBD whether the stripped oocytes were kept in the same solution with the isolated follicle cells or were placed in fresh SW lacking the follicle cells. Moreover, simply transferring control specimens with intact follicular sheaths to another SW solution 15 min after ovarian maceration did not prevent ovulation or GVBD. These results collectively indicate that the inhibition of GVBD was not due simply to the dilution of soluble cues generated by the maceration procedure.

In contrast, specimens stripped of their surrounding follicle sheath more than 40 min after removal of the follicles from the ovaries consistently showed high levels of GVBD (Figs. 7C, 8), which suggests that the stripping procedure did not block GVBD by merely increasing cell morbidity. The time at which follicle cell removal was accompanied by only a 50% level of GVBD (= the "$T_{1/2}$ inhibitory time") averaged 37 ± 12 min (n = 16 ovaries).

Figure 2. (A–D) Photomicrographs taken over a period of about 90 min during the ovulation of a single living oocyte of *Terebratalia transversa* that was obtained after macerating a ripe ovary. The sequence shows the retraction of the follicular sheath to form a cap of follicle cells that eventually dislodges from the ovulated oocyte (arrows in 2D); scale bar for A–D = 50 μm. (E) SEM of an isolated follicle that was fixed less than 5 min after maceration of the ovary; fc = follicle cells; the arrows mark the exposed end of the oocyte that was previously attached to the germinal epithelium within the ovary prior to ovarian maceration; scale bar = 50 μm. (F) SEM of a follicle that was fixed about 75 min after removal of the ovary, showing a nearly completed follicle cap (arrow) at one pole of the ovulating oocyte; scale bar = 50 μm. (G) SEM of a completely ovulated oocyte, fixed 3 h after removing the follicle from the ovary; the rectangle outlines a region similar to that shown at higher magnification in 2I; scale bar = 50 μm. (H) SEM of the cap of follicle cells (arrows) that forms at one pole of the oocyte during ovulation; scale bar = 10 μm. (I) SEM of the surface of an ovulated oocyte fixed 3 h after removing the follicle from the ovary, showing holes (arrows) in the vitelline envelope through which follicle-cell projections presumably extended prior to ovulation; scale bar = 1 μm; inset: photomicrograph of a cap of follicle cells that had become detached from the oocyte following ovulation; scale bar = 10 μm. (J) Photomicrograph of an incompletely ovulated oocyte with a follicle cell sheath that was in the process of being retracted at the time of fixation (about 70 min after removal of the follicle from the ovary); germinal vesicle breakdown (GVBD) had begun before ovulation was completed, as evidenced by the irregular outline of the GV; scale bar = 50 μm.



(K) Photomicrograph of an oocyte fixed about 75 min after removing the follicle from the ovary; the partially retracted sheath of follicle cells was dislodged from the oocyte surface during preparation of the specimen for microscopy; note that GVBD had begun, but the nucleolus (nl) is still present; scale bar = 50 μm. (L) Photomicrograph of an oocyte fixed 2 h after removal of the follicle from the ovary; both ovulation and GVBD have been completed; scale bar = 50 μm.

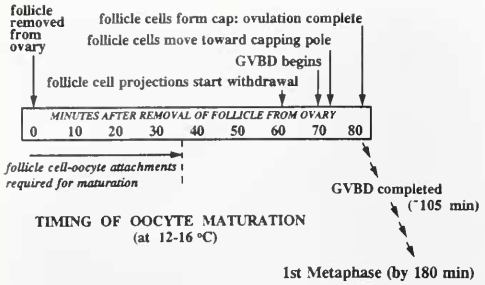


Figure 3. Summary of the timecourse of events during normal oocyte maturation.

In comparison, the $T_{1/2}$ ovulation time for cohort controls not stripped of their follicular sheath was 79 ± 18 min post-detachment of the follicles from the ovaries (n = 16), and the control groups averaged >90% GVBD by 3 h after ovarian maceration.

To determine whether gap-junction uncouplers can affect the percentage of oocyte maturation achieved, intact ovaries were immersed for 1–2 h in 12°–16°C SW solutions of 1 m*M* heptanol or 25 μM α-glycyrrhetinic acid (AGA) before being macerated. This method was chosen because pre-incubations of 30 min have been used to uncouple mammalian follicle cell–oocyte gap junctions at 37°C (Downs, 1995). After the pre-incubated ovaries were macerated, isolated follicles were soaked in the gap junction uncoupler for another 3 h. Both gap-junction uncouplers significantly reduced ($P < 0.05$) the numbers of oocytes that fully matured (*i.e.*, ovulated and underwent GVBD) (Fig. 7D, E). Moreover, the decrease was not solely due to cell morbidity, because heptanol- or AGA-treated samples that had been washed overnight in seawater had higher percentages of maturation the next morning and were able to produce normal blastulae after fertilization (pers. obs.).

Furthermore, many drug-treated oocytes underwent GVBD but failed to complete ovulation (Fig. 7F, G). Thus, compared to the 93% ± 10% level of ovulation and accompanying GVBD in six batches of controls not receiving the gap-junction uncouplers, heptanol treatments reduced GVBD to 40% ± 40% and decreased the level of full ovulation to 32% ± 39% (n = 6). Similarly, applications of the AGA uncoupler yielded 66% ± 33% and 33% ± 22% levels of GVBD and successful ovulation, respectively (n = 6), which in turn suggests that the drug treatment may have also affected the transmission of signals that enable ovulation to proceed normally.

To assess the effects of such uncoupling drugs on GVBD without incubation of the intact ovaries, isolated follicles were removed from macerated ovaries and transferred directly to 1 m*M* heptanol or 25 μM AGA. As shown in Figure 9, the percentages of GVBD in the unincubated, heptanol-treated specimens were significantly higher ($P < 0.05$; n = 6) than those attained following a 1–2 h pre-incubation in heptanol, and unincubated AGA-treated follicles exceeded the levels of GVBD displayed by controls. Pre-incubation may thus be needed to achieve optimal gap-junction uncoupling before maturation is triggered.

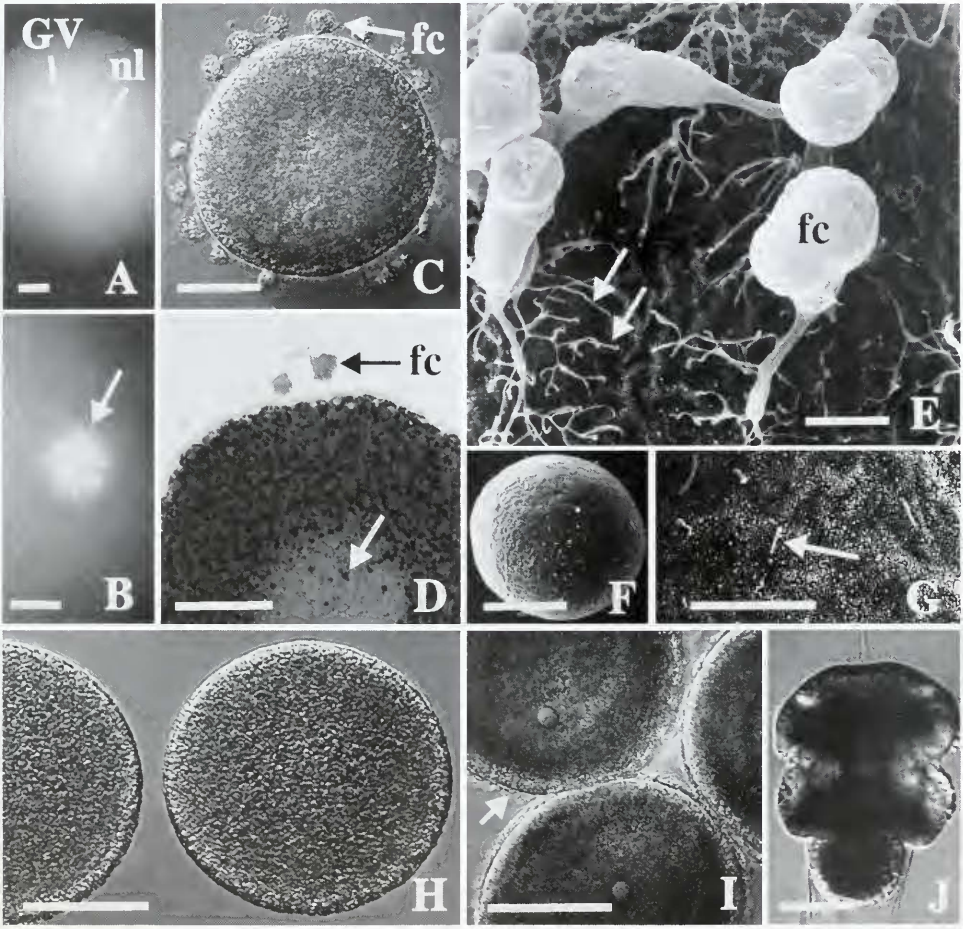
In view of these results on the effect of pre-incubation and the observation that follicles undergo morphological alterations an hour after being treated with CD, intact ovarian lobes on bisected shell valves were placed in a 10-μM SW solution of CD for 1–2 h before the pre-incubated ovaries were macerated, and the isolated follicles were immersed for another 3 h in CD (Figs. 7H, 10). After the additional 3 h in the CD solution, the percentage of GVBD was reduced to 33% ± 33% (n = 7). This maturation level was significantly lower ($P < 0.05$) than the 81% ± 30% (n = 7) GVBD level obtained in CD-treated oocytes from cohort ovaries (n = 7) that were not pre-incubated in CD but only immersed in it for 3 h after ovarian maceration, which in turn suggests that GVBD is inhibited by a prior incubation in CD that allows follicle cell–oocyte attachments enough time to be altered before oocyte maturation can be triggered.

Discussion

In vitro *maturation of* T. transversa *oocytes in relation to detachment of follicles from the ovaries*

To provide a positive stimulus for oocyte maturation, the follicle cells of many animals generate maturation-inducing factors in response to a gonad-stimulating substance (GSS) supplied by extraovarian tissues. For example, the GSS of starfish is produced in the vicinity of the radial nerves and transported to the gonads where it can cause follicle cells to secrete the maturation inducer 1-methyladenine (Meijer and Guerrier, 1984). Similarly, the gonadotropic hormones of vertebrates are gonad-stimulating substances secreted by extraovarian tissues; these hormones can cause the release of maturation-inducing steroids from follicle cells (Masui and Clarke, 1979). Alternatively, mammalian oocytes can be triggered to undergo

Figure 4. (A) Photomicrograph of a Hoechst-labeled oocyte 10 min after removal from the ovary, showing an intact germinal vesicle (GV) with a brightly fluorescent nucleolus (nl); note: the follicle cells had been removed during processing of the specimen; scale bar = 10 μm. (B) Photomicrograph of a Hoechst-



labeled oocyte 3 h after removal from the ovary, showing a circular set of chromosomes (arrow) at the metaphase plate; scale bar = 10 μm. (C) Photomicrograph taken 3 h after an oocyte was removed from the ovary and directly transferred into 10 μM cytochalasin D to block follicle cell (fc) capping. Such CD-treated oocytes nevertheless undergo GVBD; scale bar = 50 μm. (D) Epon section (1 μm) of a CD-treated oocyte at 3 h post-treatment, showing follicle cells (fc) still surrounding the oocyte, but the absence of an intact nuclear envelope, as evidenced by the mixing of nucleoplasm with cytoplasmic granules (arrow); scale bar = 50 μm. (E) SEM of a CD-treated oocyte at 3 h post-treatment, showing follicle cells (fc), which round up and acquire spindly projections with knoblike endings (arrows) starting around 1 h post-treatment; scale bar = 10 μm. (F) SEM of a CD-treated oocyte that had been subsequently washed in SW to allow the shedding of follicle cells; scale bar = 50 μm. (G) SEM of the surface of a CD-treated oocyte that had been subsequently washed in SW to allow the shedding of follicle cells; a few follicle cell projections (arrow) remain; scale bar = 10 μm. (H) Photomicrograph of two oocytes that had completed ovulation and GVBD within 2.5 h after treatment with calcium-free seawater; scale bar = 50 μm. (I) Photomicrograph of three follicles that had been treated with a 1% seawater trypsin solution for 30 min after removal from the ovary; the follicle cells (arrow) of such trypsin-treated specimens lay close to the oolemma, but eventually undergo retraction, coupled with GVBD; scale bar = 50 μm. (J) Photomicrograph of an approximately 3-day-old larva that developed from a CaFSW-treated follicle, which was subsequently washed in seawater and inseminated after GVBD; scale bar = 50 μm.

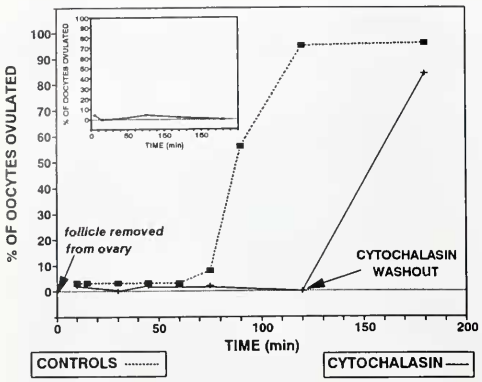


Figure 5. Follicles isolated from a macerated ovary and subsequently incubated in 10 μM cytochalasin D fail to undergo ovulation by 3 h post-treatment (inset). However, if the cytochalasin is washed out at 2 h post-treatment, essentially the same percentage of ovulation occurs in follicles previously treated with cytochalasin as in control specimens that were obtained from the same ovary but were not subjected to cytochalasin. Such findings indicate that the cytochalasin treatment only temporarily blocks ovulation and does not prevent follicle-cell capping simply by killing the follicles.

spontaneous maturation by removing their follicular sheaths, thus eliminating the inhibitory "arrestor" signal provided by the follicle cells (Cho *et al.*, 1974; Racowsky and Baldwin, 1989; Downs, 1997).

Results from this analysis of *T. transversa* fail to provide evidence either that an inhibitory substance is supplied by the follicle cells or that an extraovarian GSS is required during *in vitro* maturation. The lack of follicular inhibition of maturation is demonstrated by the failure of *T. transversa* oocytes to mature if they are mechanically stripped of their follicle cells within 30 min of being isolated from the ovaries. Similarly, gap-junction uncouplers, which would presumably prevent inhibitors from passing through the junctions connecting follicle cells to the oocyte, cause a drop in maturation rather than the increase that would be expected if maturation were arrested simply as a result of the inhibition provided by an intact follicular sheath.

Oocyte maturation in the apparent absence of an extraovarian GSS is suggested by the finding that oocytes regularly mature following maceration of just the dorsal or ventral ovaries. Thus, if a GSS is required for *in vitro* maturation, it either is produced in the ovaries or adjacent tissues, or it is somehow stored in these regions after being generated at other extraovarian sites. The requirement for a GSS is further questionable considering that oocytes continue to mature after the fluids generated by ovarian macerations are rapidly replaced with SW. Hence, if a soluble GSS is required, it either affects follicle cells almost instantaneously upon release or is active at concentrations lower than those obtained by the washing procedure used in this study. Moreover, a supernatant derived from macerated ovaries does not immediately promote high levels of maturation in intact ovaries, indicating that if a soluble GSS is indeed released by maceration, the procedure used in this study failed to extract it at a concentration capable of eliciting maturation of intraovarian follicles within 3 h after treatment.

Alternatively, *in vitro* maturation may simply depend on the physical detachment of the follicle from the ovary (Fig. 11). This possibility is consistent with the observation that ripe ovaries examined in this study seemed to lack free-floating follicles. Accordingly, reports of *T. transversa* follicles being detached from the ovarian wall (Long, 1964) might reflect follicles dislodged during specimen handling. In fully ripe females, dislodging can occur if the shell valves are not opened gently enough (pers. obs.). Judging from scanning electron micrographs of freshly isolated follicles, attached intraovarian follicles presumably possess a complete follicular sheath that shields the enclosed oocyte from external cues such as injected seawater. Moreover, immediately after removal from the ovaries, virtually all oocytes have an intact GV. Collectively, these observations suggest that an intraovarian oocyte remains arrested at prophase I as long as it is fully encased by a follicular sheath that is connected to the germinal epithelium.

Following mechanical disruption of the ovary, each detached follicle acquires an opening at one end of the follicular sheath, exposing the oocyte to seawater. In some other animals, such exposure may trigger GVBD (Stricker, 1996), presumably by altering the levels of pH, calcium, or other ions from those maintained in intact,

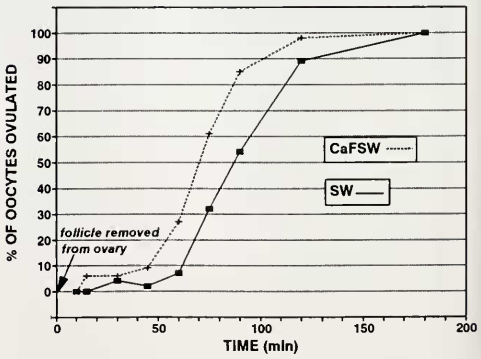


Figure 6. Typical kinetics of ovulation in oocytes obtained from the same ovarian lobes and subsequently incubated at 12°–16°C in seawater *versus* calcium-free seawater. Note the accelerated rate of ovulation in CaFSW.

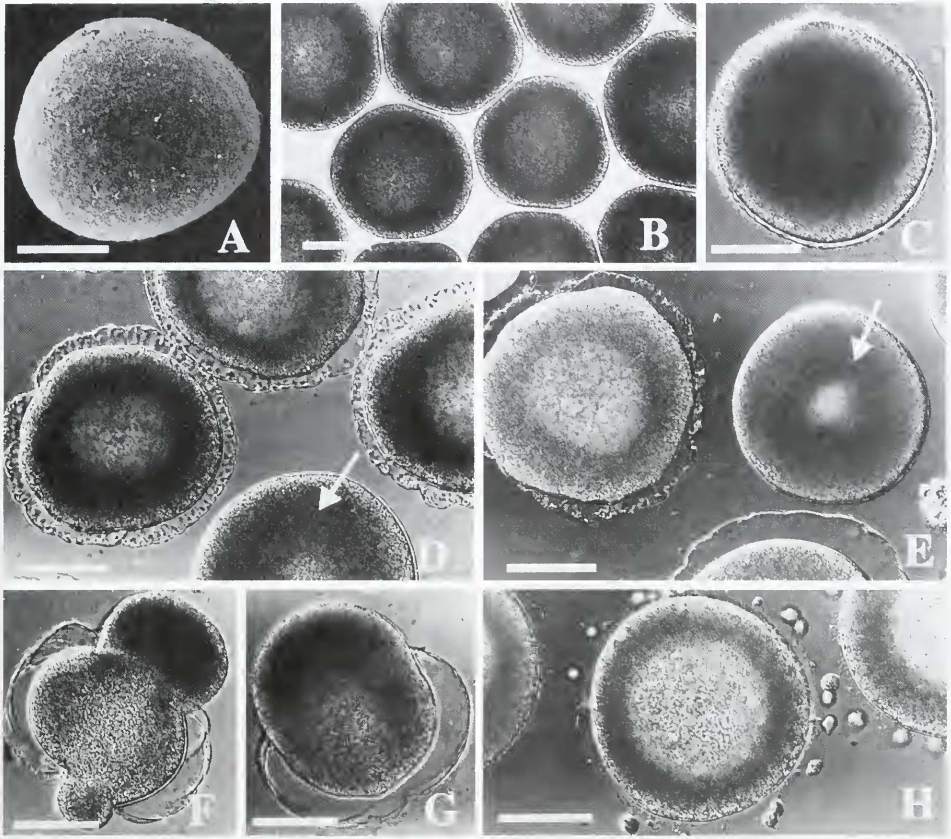


Figure 7. (A) SEM of an oocyte that had been mechanically stripped of its follicle cells about 15 min after removal of the follicle from the ovary and fixed at 20 min post-removal; scale bar = 50 μm. (B) Photomicrograph of oocytes at 3 h after removal from the ovary. Such oocytes were mechanically stripped of their follicle cells 30 min after removal from the ovary and failed to undergo GVBD, as evidenced by the intact GV in each specimen; scale bar = 50 μm. (C) Photomicrograph of an oocyte that was stripped of its follicle cells at 40 min after removal from the ovary and subsequently underwent normal GVBD; scale bar = 50 μm. (D, E) Photomicrographs of specimens that were obtained from ovaries pre-incubated for 1–2 h in the gap-junction-uncoupling drugs heptanol (D) or α-glycyrrhetinic acid (E) prior to ovarian maceration; most of the follicles obtained from such drug-treated ovaries and subsequently incubated in the drug failed to undergo GVBD by 3 h after removal of the follicles from the ovaries, although a few specimens (arrows) completed maturation; scale bars = 50 μm. (F, G) Photomicrographs of drug-treated oocytes that had completed GVBD but that failed to ovulate normally after incubating for 3 h in the gap-junction-uncoupler heptanols (F) or α-glycyrrhetinic acid (G); scale bar = 50 μm. (H) Photomicrograph of cytochalasin-treated follicles that failed to undergo ovulation or GVBD by 3 h post-treatment, when ovaries were pre-incubated in 10 μM CD for 1–2 h prior to the isolation of the follicles by ovarian maceration; compare with Fig. 4C, D where the ovaries were not pre-incubated in cytochalasin prior to maceration but the oocytes subsequently matured while in cytochalasin; scale bar = 50 μm.

attached follicles. In *T. transversa*, however, seawater exposure by itself does not cause *in vitro* maturation, since oocytes stripped of their follicle cells less than 30 min after removal from the ovaries fail to undergo GVBD even when they are incubated overnight in seawater. Thus, the exposure to seawater, some other factor

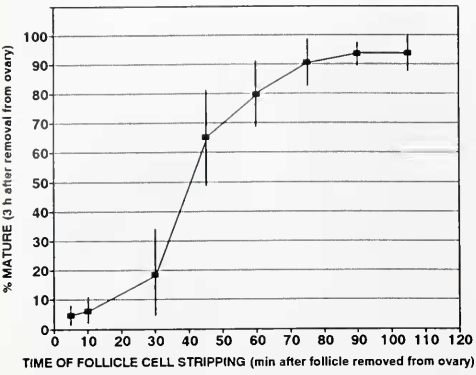


Figure 8. Mechanically removing follicle cells by means of a Nitex filter up to 30 min after obtaining the follicles from the ovaries yields low percentages of maturation by 3 h, whereas stripping follicle cells more than 40 min after obtaining the follicles from the ovaries does not significantly prevent maturation. These findings are consistent with the view that a maturation-inducing substance is transferred from the follicle cells to the oocyte within about 30–40 min after removing the follicle from the ovary. Vertical line = standard error of the mean; n = 16 for each time-point.

elicited by follicular detachment, or a combination of the two apparently triggers the production of an as-yet unidentified substance in the follicle cells, which in turn leads to oocyte maturation.

Although simple detachment of the follicle from the ovary seems capable of triggering oocyte maturation *in vitro,* a GSS may nevertheless function during normal spawnings in the field. Thus, in response to appropriate environmental cues such as daylength, temperature, or biomass load in the surrounding seawater, a GSS discharged by cells in the ovaries or adjacent tissues could trigger the detachment of follicles, perhaps by causing constrictions at sites where follicles connect to the germinal epithelium. Such a GSS-induced pathway would presumably take longer to detach follicles than does mechanical disruption, and could be fully replaced by the maceration procedure during *in vitro* trials. As indirect evidence for this view, overnight exposure of intact ovaries to an ovarian supernatant yields higher percentages of oocyte maturation than are observed in response to seawater alone, suggesting that some component of the supernatant helps to promote the detachment of the follicles in otherwise intact ovaries that are incubated for prolonged periods.

Ovulation-GVBD relationships and the roles of follicle cell-oocyte attachments during maturation

Following maceration of the ovaries, fully formed oocytes of *T. transversa* undergo ovulation and GVBD in tandem. Thus, ovulated oocytes subsequently complete GVBD, and GVBD is not accomplished without prior shedding of the follicular sheath, suggesting that such processes may be causally linked. However, GVBD can be uncoupled from ovulation by experimental manipulations such as the application of cytochalasin D, which blocks follicle-cell capping but nevertheless allows GVBD to proceed. Conversely, if freshly collected specimens are mechanically stripped of their follicle cells within 30 min after removal from the ovary, GVBD typically fails to occur even though follicle cells are no longer present. Collectively, these findings suggest that although ovulation is normally followed by GVBD during *in vitro* maturation, successful removal of the follicular sheath is neither necessary nor sufficient to trigger GVBD in experimentally manipulated specimens.

The timing of ovulation can also be altered by CaFSW or trypsin treatments, which may simply accelerate follicle-cell capping by weakening follicle cell–oocyte attachments; incubating ovaries in seawater for more than 3 h prior to maceration may have the same effect. The converse of this mechanism could not, however, account for the more protracted ovulation obtained with oocytes from

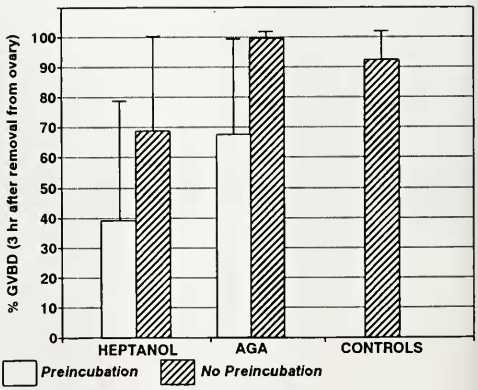


Figure 9. Pre-incubating ovaries in gap-junction-uncoupling drugs (1 m*M* heptanol or 25 μM α-glycyrrhetinic acid [= AGA]) for 1–2 h prior to ovarian maceration significantly reduces the percentages of maturation that are achieved after a subsequent 3-h treatment of the isolated follicles in the drug. Alternatively, follicles that are freshly obtained from macerated ovaries without a prior incubation in the drug before maceration do not show significantly lower percentages of maturation when subsequently incubated in the gap junction uncoupler for 3 h. Since the gap junction uncouplers require some time to disrupt intercellular communication, these findings are consistent with the view that functional follicle cell–oocyte attachments are required for about 30–40 min after follicular removal from the ovary in order for maturation to proceed. Controls are follicles isolated from the same ovaries as used for the drug treatments but not subjected to either of the gap-junction uncouplers. Vertical line = standard error of the mean; n = 6 for each treatment and control.

dorsal ovaries, unless oocytes of equal size in both types of ovaries somehow have more firmly attached follicle cells in the dorsal ovaries. Alternatively, separating the two shell valves removes the ventral ovaries from the lophophore and viscera, because these organs invariably remain attached to the dorsal valve. Oocytes obtained from separated ventral valves are thus no longer situated near the internal organs to receive the kinds of cues—either non-specific or specific—that apparently delay the ovulation of oocytes derived from isolated dorsal valves.

Evidence from experimental removal of follicular sheaths suggests that the follicle cells of *T. transversa* transport a maturation-inducing substance to the oocyte by about 30–40 min after the follicle is detached from the ovary. This timing also coincides with the kinetic data obtained from time-lapse video studies in that follicle cells are normally still attached to the oocyte 30–40 min after follicular removal from the ovary, and GVBD does not begin until about 70 min post-removal. Is the putative maturation-inducing substance actually transmitted from the follicle cells at around 30–40 min post-detachment of the follicle, or does an intact follicular sheath need to be continuously present for 30–40 min? These possibilities

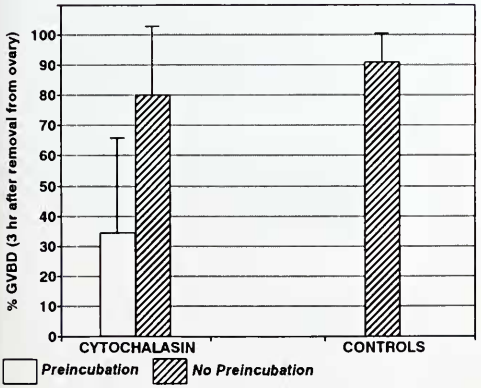


Figure 10. Preincubating ovaries in 10 μM cytochalasin for 1–2 h prior to ovarian maceration significantly reduces the percentages of maturation that are achieved after a subsequent 3-h treatment of the isolated follicles in the drug. Alternatively, follicles that are freshly obtained from macerated ovaries without a prior incubation in cytochalasin before maceration do not show significantly lower percentages of maturation when subsequently treated with cytochalasin for 3 h. Because cytochalasin treatments take about 1 h to alter follicle cell morphology, these findings are consistent with the view that functional follicle cell–oocyte attachments are required for 30–40 min after follicular removal from the ovary in order for maturation to proceed. Controls are follicles isolated from the same ovaries as used for the cytochalasin treatments but not subjected to cytochalasin. Bars = standard error of the mean; n = 17 for the "no pre-incubation" treatment, and n = 7 for the "pre-incubation treatment" and controls.

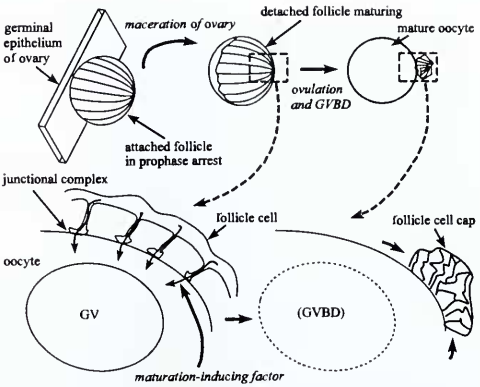


Figure 11. Diagram of the key role that follicle cell–oocyte attachments are believed to play during oocyte maturation, based on time-lapse video analyses and experimental manipulations.

cannot be distinguished on the basis of the experiments conducted in this investigation. However, unlike some other species in which a soluble maturation-inducing substance can be secreted externally to trigger maturation throughout the surface of the oocyte (Patino and Purkiss, 1993), in *T. transversa* the follicle cells apparently must be attached to the oolemma, judging from the fact that freshly collected oocytes that had been separated from their follicle cells did not undergo GVBD, even when kept in the fluids containing the stripped follicle cells. Similarly, gap junction uncouplers can at least partially prevent GVBD, even though follicle cells remain present near the non-maturing oocytes, following the apparent disruption of the follicle cell–oocyte junctions by the drugs.

The junctional complexes observed in *T. transversa* follicles have not been definitely identified as gap junctions, so the inhibitions obtained with the uncoupling drugs could be due to nonspecific effects rather than to actual disruptions of the junctions. In many cases, gap junctions can be distinguished by various ultrastructural characteristics, especially in the presence of lanthanum tracers (Larsen and Wert, 1989). Some invertebrate tissues, however, contain junctions that do not resemble conventional gap junctions but nevertheless allow small molecules to pass between neighboring cells (Germain and Anctil, 1996). Thus, unequivocal proof that the junctional complexes in *T. transversa* follicles possess the physiological properties of communicating junctions (as suggested by the drug tests) will necessitate functional analyses based on microelectrode investigations of ionic coupling (Gilula *et al.*, 1978) or dye-tracer studies using injected fluorescent probes (Browne *et al.*, 1979; Cerada *et al.*, 1993). Unfortunately, the vitelline envelope sur-

rounding *T. transversa* oocytes prevents easy access to the oolemma for electrophysiological recordings and hinders microinjection of fluorescent dyes by methods that have been successful with the oocytes of other marine invertebrates (Stricker *et al.,* 1992b, 1994b; Stricker, 1996).

In any case, the need for intact follicle cell–oocyte attachments is indirectly indicated by two other sets of results. First, gap-junction uncoupling drugs, which take about 30 min to block intercellular communications at 37°C (Downs, 1995), fail to block GVBD if *T. transversa* follicles are placed directly in the drug without preincubating the ovary prior to maceration. Maturation percentages are reduced, however, if the ovary is immersed in the drug for 1–2 h before maceration, particularly in the case of heptanol, which may use a more rapid mechanism of uncoupling than does AGA. Similarly, CD treatments typically fail to prevent GVBD when detached follicles are placed directly into the CD solution without an incubation in the drug before ovarian maceration. However, when ovaries are pre-incubated in CD, thereby allowing the drug more time to alter follicle-cell morphology before the ovary is macerated, CD significantly reduces GVBD levels. Thus, two very different kinds of drug treatments point toward the need for intact follicle cell–oocyte attachments and are consistent with the view that maturation can be inhibited if follicle cell–oocyte attachments are altered within 30–40 min after the follicle is detached from the macerated ovary.

Comparative biology of oocyte maturation in brachiopods

In the inarticulate brachiopod *Glottidia pyramidata,* oocyte maturation cannot be triggered by mechanical disruption of the ovaries, because such treatments tend to lyse oocytes (Freeman, 1994). Follicle-cell-free oocytes are obtained after repeatedly washing small pieces of *G. pyramidata* ovaries in CaFSW, but such oocytes fail to undergo GVBD (Freeman, 1994). It is not fully clear if this lack of maturation in the CaFSW-treated oocytes is simply due to a precocious removal of follicle cells prior to their delivery of a maturation-inducing signal, as has been observed in mechanically stripped follicles of *T. transversa.* However, following the CaFSW washes of *G. pyramidata* ovaries, "the follicular epithelium around the oocyte retracts" (Freeman, 1994, pg. 265), which implies that such CaFSW-treated specimens actively ovulate. Therefore, unlike the oocytes of *T. transversa,* which complete GVBD after ovulation, those of *G. pyramidata* are apparently capable of undergoing ovulation without concomitant GVBD.

Oocyte maturation can be elicited, however, if pieces of *G. pyramidata* ovaries are incubated with a soluble extract of the lophophore, although extracts from other tissues, including the gonads, fail to trigger GVBD (Freeman, 1994). Similar incubations with membrane-permeant forms of cAMP also cause maturation, which suggest that the follicle cells of *G. pyramidata* possess a cAMP-based signaling pathway that is normally triggered by a lophophore-derived GSS to stimulate the secretion of a maturation-inducing substance (Freeman, 1994).

Whether *T. transversa* oocytes can be induced to mature in a similar fashion is difficult to determine because there is no reliable way of obtaining pieces of ripe ovaries without also triggering maturation through the mechanical detachment of the follicles. Hence, such studies would have to ensure that the experimental solutions gained access to the follicles within intact ovaries, and that maturation was not simply elicited by dislodging follicles from the ovaries. However, based on the fact that the simple maceration of *T. transversa* ovaries routinely triggers oocyte maturation, it follows that in this species (i) any GSS that might be produced by *T. transversa* is not restricted to the lophophore, and (ii) *in vitro* maturation does not require treatments with lophophore extracts or exogenously supplied cAMP, as has been described for *G. pyramidata.* The underlying causes for such apparent differences between the two species remain obscure.

Additional analyses of brachiopod ovulation and GVBD are essentially lacking, and the timing of these events relative to spawning and fertilization in the field are generally not known (Long and Stricker, 1991). The few observations that have been recorded indicate a range of possibilities. In the articulate brachiopod *Terebratulina retusa,* the follicle cells tend to be shed some time after spawning but apparently just prior to fertilization (James *et al.,* 1991a, b). In the inarticulate species *Crania anomala,* freshly spawned oocytes already lack follicle cells prior to fertilization (Nielsen, 1991). In contrast, spawned oocytes of the articulate brachiopod *Calloria inconspicua* retract their follicle cells after being fertilized (Chuang, 1996).

On the rare occasions when spawning has been observed in *T. transversa,* the oocytes contained an intact GV and a follicular sheath, but unlike what has been determined here for laboratory cultures, the exact maturation state of naturally spawned oocytes at the time of fertilization has not been ascertained (Long, 1964; Long and Stricker, 1991). Moreover, although long-term storage of adult *T. transversa* females in laboratory aquaria with running seawater often leads to an accumulation of fully grown, immature oocytes (Reed, 1987), the underlying mechanism for this apparent inhibition of spawning and maturation remains unknown. Thus, further comparisons between the laboratory microenvironment and the normal subtidal habitat may provide important clues about the natural stimuli for maturation (Rokop, 1977). Based on the *in vitro* analyses of *T. transversa* reported here, *in situ* investigations should consider the role that follicle

cells, and particularly their attachments to the oocyte, play in maturation and fertilization in the field.

Acknowledgments

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